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Histochemical activity of 5-4-isomerase-3-B hydroxy steroid dehydrogenase in the ovary of the viviparous mexican lizard *Sceloporus mucronatus* (Reptilia: Prhynosomatidae) and interelationship with progesterone levels during pregnancy

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ABSTRACT

Objective: To relate the histological characteristics and histochemical Δ^{5-4}-isomerase-3 beta hydroxy steroid dehydrogenase (Δ^{5-4}3β-HSD) activity of the corpora lutea (CL) and the atresic vitellogenic follicles (AVF) with progesterone (P_4) plasma concentrations in three different times of gestation (early, medium and late) in the viviparous lizard *Sceloporus mucronatus* (*S. mucronatus*). **Methods:** The histological characteristics as well as histochemical activity of Δ^{5-4}3β-HSD of the CL and AVF and their relationship with plasma P_4 levels were studied during three different times of pregnancy of the viviparous lizard *S. mucronatus*. **Results:** Corpora lutea develops during the first third of gestation. In second third, the luteal tissue reaches maturity and starts the first regressive changes. The last third of gestation was characterized by a considerably advance in the luteolysis. Activity of Δ^{5-4}3β-HSD was observed in he luteal cell mas. The activity of this enzyme were high during the first third and scantle activity was detected in the last third. Even though atresic vitellogenic follicles are found throughout the whole period of gestation, Δ^{5-4}3β-HSD activity is very low in relation with showed by CL and does not change significantly in the studied period of time. Another hand, we observed a direct relationship among the histological aspect of the corpus luteum, Δ^{5-4}3β-HSD activity and progesterone levels. **Conclusions:** These observations suggests that the corpus luteum is the most important source of ovarian progesterone (P_4) during pregnancy and that the participation of the AVF in the production of this hormone is little or non-existent.

1. Introduction

The corpora lutea (CL) is a transitory endocrine gland present in the ovary of pregnant vertebrate females[1]. Several authors agree that in reptiles the CL is the most important source of progesterone (P_4) during pregnancy[2]. However, other authors have found indirect evidence suggesting that the atretic vitellogenic follicles (AVF)[3,4], the adrenal gland (AG)[5] and the corio-allantoic placenta (CAP)[3,6] are able to produce P_4 during some stage of pregnancy. In many reptiles, the AVF react little to Δ^{5-4}-isomerase-3β hydroxy steroid dehydrogenase (Δ^{5-4}3β-HSD). However, some species show high activity of this enzyme in large AVF, suggesting that it could be participating in the steroidgenic activity of the ovary[7]. The Δ^{5-4}3β-HSD is an enzyme that converts pregnenolone in P_4 or dehydroepiandrostenedione in androstenedione[8]. In several lizard species as: *Mabuya carinata*[9], *Chalcide chalcides*[10] *Barisia imbricata imbricata*[11], the activity of this

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enzyme in the CL is directly related with levels of circulating P_4.

Sceloporus mucronatus (S. mucronatus) is a viviparous lizard with fall reproduction. Vitellogenesis occurs during summer and ovulation occurring in late September and October[12]. Females remain pregnant throughout the winter and offspring is born in April of the next year[13]. There exist several studies about histological changes occurring in the ovary of *S. mucronatus* during its reproductive cycle[4,12-14]. It has been observed that the CL remains present throughout the whole gestation period[4,12]. The luteal development occurs during the first two months of pregnancy and the first luteolitic changes take place in the second part of gestation[4]. The AVF are present during vitellogenesis as well as during gestation[11]. Villagrán-Santa Cruz has observed an increase in number and volume of the AVF in the second stage of gestation, coinciding with the degeneration of the CL[4]. As such, the author suggests that the AVF might assist or replace the CL in the production of P_4 during the last stage of gestation[4] even though there is yet no evidence of their potential steroid production. This work aims to relate the histological characteristics and histochemical $\Delta^{5-4}3\beta$-HSD activity of the CL and the AVF with P_4 plasma concentrations in three different times of gestation (early, medium and late) in the viviparous lizard *S. mucronatus*.

2. Materials and methods

2.1. Animals

Adult *S. mucronatus* females were collected [snout-vent length: (80±7) mm; weight: (22.3±7.6) g] in Tepeapulco, Hidalgo State (19°52′N, 98°24′W), México, during the first half of October (n=4), November (n=6), and December (n=5) of the year 2010. The next year (2011), the collection continued during the first fortnight of February (n=6) and April (n=7). Immediately after capture a blood aliquot of 200-220 μL was obtained from each lizard by cardiac puncture using an heparinised syringe and then centrifuged during 1 min at 6 000 r/min. Plasma was decanted and frozen in CO solid and transported to the laboratory, where samples were stored at -40 °C until radioimmunoassay for P_4 were performed. All aliquots were obtained between 9.00-13.00 h. On the day of capture, the lizards were marked by toe clipping for individual identification and were transported to the laboratory where they were weighed and measured. The next day, the organisms were anaesthetized by intraperitoneal injection with sodium pentobarbital (6.3 mg/mL, 10 μL/25 g[15] and submitted to surgery. The ovaries were localized and the number of CL and AVF was registered. Two samples of each structure were immediately removed from each female. One CL and one AVF were included in Tissue-teck (TRIANGLE BIOMEDICAL SCIENCES) and immediately frozen in a mixture of acetone and solid CO_2. They were stored at -40 °C until histochemical detection of $\Delta^{5-4}3\beta$-HSD activity. The other CL and AVF were fixed in 10% neutral-buffered formalin. The lizards were sutured and deposited in individual terraria. Every female received an injection (IM) of 5000 I.U. on the day of surgery of Penicillin G and following two days 2.5 mg of streptomycin. The lizards were maintained in the laboratory for three days to recuperate. Afterwards, the animals were kept during four weeks under natural conditions inside the greenhouse of the FES-Iztacala (19°36′N; 98.5°11′W; 2 250 m altitude), providing them with water and food *ad libitum* (mealworms, domestic crickets and grasshoppers). One month after surgery, the lizards survivors (n=19) were liberated on the same site where they were previously collected and the kill lizards were deposited in the collection of the Instituto de Biología UNAM, México City (CNAR IBH 16411-16416). All experimental procedures were approved by the Bioethical Committee of the FES Iztacala UNAM.

2.2. Determination of $\Delta^{5-4}3\beta$-HSD activity

Three at five days after surgery, sections of 12 μm thicknesses of the CL and AVF (previously embedded in tissue-teck) were performed in a cryostat maintained at -20 °C and processed according to the technique described by Levy *et al*[16]. The slices were incubated at 37 °C during 1 hour in a medium containing 40 mg Nicotinamide Adenine Dinucleotide and 20 mg Nitro Blue Tetrazolium dissolved in 40 mL phosphate buffered solution (pH 7.4). Two mg dehydroepiandrostenedione dissolved in 0.5 mL N,N-dimethylformamide was added a substrate. Control sections of mouse ovaries and kidneys were incubated with or without substrate. All chemicals used in this study were supply by Sigma Chemical (S. Louis, MO).

2.3. CL histology

Corpora lutea and AVF were washed 24 h in running water and gradually dehydrated with alcohol. They were cleared with xylene and included in paraffin wax[17]. Serial sections (7 μm of thick) were elaborated and stained with haematoxylin and eosin[17]. Diameter of CL, diameter of the luteal cell mass (LCM) an thecal layer were measured with Motic Software.

2.4. Progesterone assay

A progesterone assay was performed using a commercial radioimmunoassay kit (Coat. A-Count, Progesterone, Diagnostic Products Corporation, C.A. 90045). 125I-labelled progesterone was used as tracer; plasma was non-diluted and non-extracted; the antiserum was P_4 specific. The procedure utilized was previously described[11]. Antiserum was specific to progesterone. Inter- and intra-assay coefficients of variation were 9.6 and 6.7 respectively. Sensitivity of assay was 0.02 ng/mL.

2.5. Statistics

One way analysis of variance (ANOVA) was used to analyze the variations in progesterone plasma levels as well as the number of the CL and AVF along gestation. Post-hoc test was carried out using Tukey method to determine differences along the studied times, P<0.05 was used to asses statistical significance. Statistic analyses were performed using software Sigma Stat 2.3 for Windows.

3. Results

The females collected in October did not present eggs in their uterus, but they did present vitellogenic follicles. Uterine eggs as well as CL were found in 2 to 6 females collected in November and all females collected in December. As a result, lizards collected in October and November were not used for the aim of this work; only lizards collected during December, February and April. There were also no significant differences between number of CL ($F_{(2,17)}$=0.485, P>0.062 4) neither between number of AVF ($F_{(2,17)}$=0.353, P>0.708) among collected females utilized for this study.

The general histological characteristics of the CL and the AVF of *S. mucronatus* coincide with previous descriptions for this species[4,12] and other lacertilians[11]. The histological characteristics most striking of CL and AVF were as follow.

3.1. Corpus luteum in early pregnancy

Four of the five females collected in December presented a cavity in the CL (Figure 1A), while only one female showed a compact structure of luteal tissue. The granulosa cells join together to form a multilaminar structure and differentiate into a luteal cells mass (LCM). The LCM constitutes of ovoid cells with one or two nucleolus and acidophilic cytoplasm. The thecal tissue was well differentiated into theca intern and theca extern, separated by largeness blood vessels (Figure 1A). The histochemical activity of Δ^{5-4}3β-HSD was observed in the LCM and the theca, however, the greater activity was found in the LCM (Figure 1B). In the theca was observed only some formazan granules (Figure 1B).

3.2. Corpus luteum in medium pregnancy

The CL of two females presented characteristics of endocrine active gland. The LCM cells presented acidophilic cytoplasm, a vesicular nucleus with one or two nucleolus and the thecas were well differentiated and separated by prominent blood vessels (Figure 1C). On the other hand, the CL of the remaining four females showed signs of degeneration. They had spherical cells containing a lipid vacuole in the cytoplasm and a peripheral condensed nucleus, flat shaped ("ring cells") (Figure 1D). Moreover, in one female the CL presented infiltration of connective tissue. Δ^{5-4}3β-HSD activity was only found in the MCL (Figure 1E) and in higher amount than observed during early pregnancy.

3.3. Corpus luteum during late pregnancy

Degeneration was more obvious. The CL of all females presented a drastically reduced diameter and the MCL showed abundant infiltration of connective tissue septa and fibroblasts as well as some small intercellular gaps (Figure 1F). Δ^{5-4}3β-HSD activity has diminished drastically and formazan granules are homogeneously dispersed throughout the MCL (Figure 1G).

3.4. Atretic follicles

Atretic vitellogenic follicles were found in all collected females except in one lizard captured in the first stage of pregnancy and one captured in the last stage of pregnancy. The AVF obtained during the first and second third of gestation, we observed that the theca contains many fibroblasts, blood vessels and acidophilic intercellular material. The oocyte has been invaded by follicular cells and presents some vitellin residuals being absorbed (Figure 2A). Some AVF in the first third of gestation showed residuals of the zona pellucida. On the other hand, the AVF in the last third of gestation showed that invaded follicle cells have adopted an elliptic shape with signs of hypertrophy. The nucleus is flat shaped and peripheral while the cytoplasm presents a large vacuole. As a result, the interior of the AVF adopts a reticular aspect (Figure 2B). There was very little Δ^{5-4}3β-HSD activity in all AVF during the three

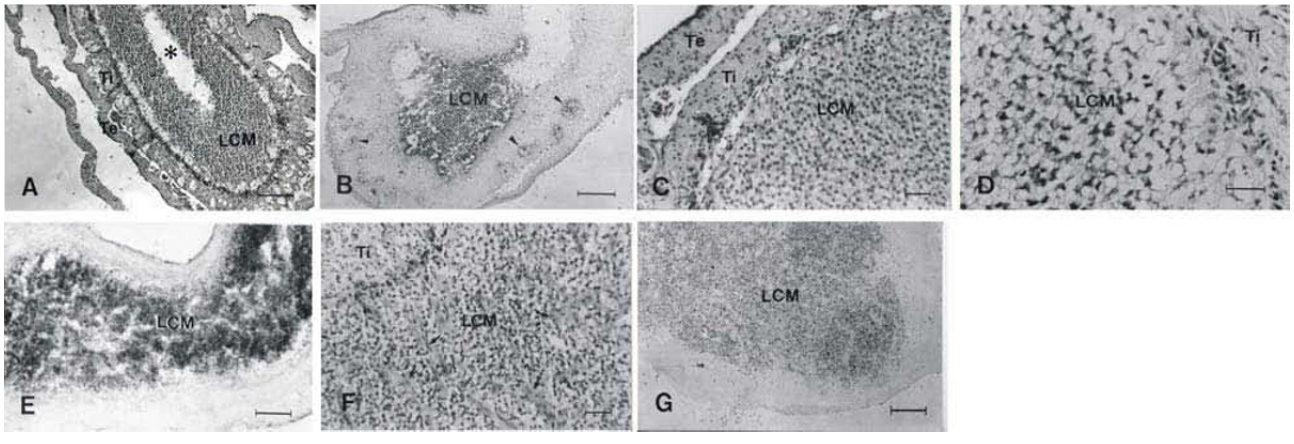

Figure 1. Corpus luteum in different times of pregnancy of *S. mucronatus*.
A) Histological section of the corpus luteum in early pregnancy. Note the presence of a central cavity (*) surrounded by LCM and the separation of Ti and Te by prominent blood vessels. Scale bar 100 μm, B) Histochemical activity of Δ^{5-4}3β-HSD in corpus luteum in the first third of pregnancy. Observe that major activity is presented in the LCM and that there are only some deposits of granules in the theca (#). Scale bar 100 μm. C) Mature corpus luteum in the middle gestation. Note that the LCM forms a complete structure consisting of cells with ovoid nucleus and Ti and Te separated by prominent blood vessels (▽) Scale bar 100 μm. D) Corpus luteum in the middle pregnancy (at time of the beginning luteolysis). Note that the LCM is formed by cells in a ring. Scale bar 50 μm. E) Histochemical activity of Δ^{5-4}3β-HSD in corpus luteum during the late pregnancy. Note that the presence of formazan granules is more abundant in comparison with corpus luteum in the first third. Scale bar 100 μm F) Histology of corpus luteum in the late gestation. Note the abundant infiltration of connective tissue (◆) in the LCM, characteristic for luteolysis. Scale bar 50 μm. G) Histochemical activity of Δ^{5-4}3β-HSD in corpus luteum during the late gestation. The number of formazan granules has diminished considerably. Scale bar 100 μm. LCM: Luteal Cell Mass; T: theca; Ti: Theca interna; Te: Theca externa.

studied phases of pregnancy. The formazan granules were observed mainly inside the oocyte during reabsorption (Figure 2C).

3.5. Progesterone

The P_4 assay was validated by parallel comparison of a serial dilution of pooled plasma from pregnant females of *B. imbricata* with a standard curve (r=0.98). P_4 levels in pregnant females of *S. mucronatus* were low during the first part of gestation [(0.48±0.15) ng/mL)], while a significant increment was detected in the second part ($F_{(2, 17)}$=33.8, P<0.001; 2.65±0.36 ng/mL). A significant reduction was observed in the last part of gestation (0.36±0.09 ng/mL). However, no statistically significant differences were found between the first and last third of gestation (Table 1). The values were obtained using GAMBYT software.

4. Discussion

Estrada *et al*. reported that the start of ovogenesis in June and ovulation occur in October for the population of *S. mucronatus* in the Ajusco National Park[11]. However, the population studied in this paper ovulation occurred at the end of November and beginning of December. Variability in the beginning of gonadal recrudescence has been observed in several lizard species (*S. gramicus*[18,19], *S. torquatus*[22,23], *B. i. imbricata*[10,20-21]. In particular, has been observed that males of *S. mucronatus* originating from different populations show differences in the beginning of gametogenesis[24], therefore it is not exceptional that females of this species to show also variations in the beginning of ovogenesis. Consequently, also could be variations in period of ovulation between populations from different latitudes. Several factors are important as controllers of reptilian reproductive activity such as temperature, photoperiod, precipitation and food availability. These factors are related with latitude and altitude on are located the populations[25]

The histological characteristics of the CL and AVF observed in the population of Tepeapulco are similar to those described by Villagrán-Santa Cruz[4]. Luteal development occurred during the first third of pregnancy and the initial degenerative changes were observed during the second part of gestation. Histochemical determination of activity of enzymes involved in steroid synthesis is a widely accepted indirect method to indicate the capacity of a tissue to synthesize sexual steroids[8]. In this study, we found that the CL presented a strong activity of Δ^{5-4}3β-HSD when the CL showed an appearance of mature gland. Moreover, during luteolysis Δ^{5-4}3β-HSD activity decrease drastically. Moreover, the activity of this enzyme had a positive relationship with histological appereance and P_4 levels throughout gestation. Similar changes have been observed for other lizard species as *Sceloporus cyanogenys*[26]; *Chamaleo pumilus* pumilus[27] and *B. i. imbricata*[11]. Villagrán-Santa Cruz observed an

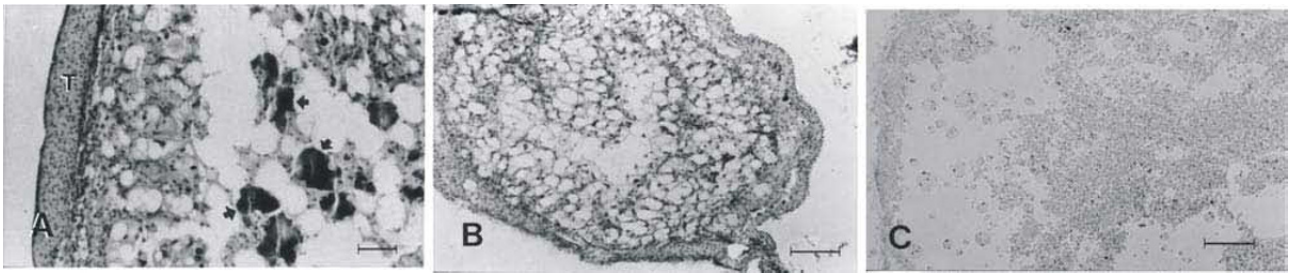

Figure 2. Atresic vitellogenic follicles (AVF) of *Sceloporus mucronatus* at different times of pregnancy A) Histological section of AVF in the early pregnancy. Note the presence of vitellin residuals surrounded by follicular cells (#). Scale bar 100 μm B) Histology of AVF in the late pregnancy. Note that the follicle presents a reticular aspect formed by vesicular cells. Scale bar 100 μm. C) Histochemical activity of Δ^{5-4}3β-HSD of AVF in the late pregnancy. Observe the very low quantity of formazan granules in comparison with the observed quantity in corpora lutea. Scale bar 100 μm.

Table 1
Corpora lutea and atretic vitellogenic follicle characteristics and progesterone levels in *S. mucronatus* pregnant females.

Pregnancy stage	Corpus luteum (mean±SD)		Atretic vitellogenic follicles (mean±SD and Δ^{5-4}3β-HSD activity [a])	Progesterone [b](Mean±SEM)
	developing (Δ^{5-4}3β-HSD activity [a])	degenerating (Δ^{5-4}3β-HSD activity [a])		
First third (n=5)	6.7±1.6		3.0±2.2	0.48±0.15
	(++ → ++++)	-	(±)	
Second third (n=6)	7.1±1.9		2.5±1.0	2.65±0.30*
	(++++++)	(++++++)	(±)	
Last third (n= 7)	6.1±1.7		3.4±2.4	0.36±0.09
	-	(++)	(±)	

[a]=Reaction intensity was symbolized in a gradient from minimal (±) to maximal (++++++); [b]ng/mL; [n]number of lizard collected; *significant differences $F_{(2, 17)}$=33.8, P<0.001.

increase in the number of AVF during the second part of pregnancy of *S. mucronatus*[4]. However, in this work, no significant differences between AVF numbers were found, and in some females these structures were even absent. The same author notes that the AVF of *S. mucronatus* present characteristics of a secretor structure[4]. The histological slices of the AVF from the second and last third of pregnancy showed a reticular aspect, similar to that described by Villagrán-Santa Cruz[13]. However, the activity of $\Delta^{5-4}3\beta$-HSD throughout pregnancy is limited in comparison with the activity observed in the CL. Moreover, there was not of increase in the activity of this enzyme in the AVF obtained in the second and last third of pregnancy.

We concluded that these observations show that the most important source of progesterone during pregnancy in *S. mucronatus* are the CL and that the participation of the AVF is little or non-existent.

Conflict of interest statement

The authors declare that they have no competing interests.

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